Fe and Zn K-edge XAFS Investigation of the Relationship Between the Ligand Structure and Enzymatic Activity of Endothelial Nitric Oxide Synthase

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Introduction: Ultra dilute Fe and Zn K-edge XAFS experiments have been performed on endothelial nitric oxide synthase (NOS) in frozen solution. This enzyme is found in the endothelial lining of blood vessels, and synthesizes nitric oxide NO. This small volatile molecule is needed for regulation of the diameter of blood vessels, and, as such, is crucial for the regulation of blood pressure. All major risk factors for arteriosclerosis, such as smoking, dyslipidemia, diabetes, hypertension and hyperhomocysteinema, have been shown to correlate with in vivo deficiency of NO. NOS are only active in dimeric state and require a special pterin (BH4) as a cofactor. In absence of BH4, NOS is known to produce large quantities of superoxide radicals, which act as strongly atherosclerotic agents. In endothelial NOS, both NO and superoxide are known to be produced at the heme domain of the enzyme. Therefore, the problem of the role of BH_4 in enzymatic activity of NOS is central to the development of arteriosclerosis.

Each NOS monomer consists of a flavin-containing reductase domain and a heme domain. The structure of the heme domain has been published in the form of crystallographic data, because a subfraction containing the heme structure has been dimerized and crystallized for X-ray diffraction studies. In contrast, the complete NOS have not been crystallized, and information on the structure of the fully functional dimer was sorely lacking so far. The role of the cofactor BH_4 was not understood.

The sample preparation required for our experiments is highly involved since NOS preparations must be obtained in absence of BH_4. This was achieved by genetic transfection of adenoviruses with DNA sequences for NOS, bringing these to expression and harvesting and purifying the NOS material. It is free of BH_4 which adenoviruses cannot produce themselves. This difficult and time-consuming preparation results in minute quantities (250 microliters) of very concentrated protein. Since every huge (260 kDA) NOS dimer contains only two heme groups, the actual iron concentration is still very low ([Fe]=250 uM in final sample after addition of cofactors and substrate), putting strong requirements on sensitivity of the EXAFS experiments. Even more challenging is Zn, which has a twofold lower concentration, since only a single Zn atom is found per dimer.

The NOS enzyme was investigated in two shifts in May and October of at beamline X9B. This beamline is specifically designed for experiments in very small and dilute samples as encountered in biological materials. It combines crucial ingredients, such as a focused X-ray beam, the liquid helium cryostat and the new Canberra 13-element Ge detector. The performance of this setup was so good that we were able to collect high-quality Fe and Zn K-edge XAFS data for the first time.

Although the EXAFS analysis of the Zn-spectra are not completed, a number of important conclusions can already be drawn at this stage

1. The Fe-EXAFS data have shown that the liganding structure of NOS heme closely resembles that of Cytochrome P450 (a well investigated heme enzyme which has been been crystallized). In particular, the presence and distance of a characteristic sulphur ligand in axial position could be confirmed.
2. It was verified that each NOS dimer contains roughly two iron atoms and a single zinc atom. The ligand structure of the Zn is very similar to that found in the crystallized dimers of the heme-subunits. It shows that, in fully functional NOS dimers, the individual monomers are are oriented head to head (i.e. that the heme domains are adjacent).
3. Incubation of NOS with BH_4 does not induce observable changes in the ligand structure of the heme. This observation is compatible with the fact that the binding site for BH_4 has considerable distance to the heme (as shown by crystallographic data from the heme-dimer).
4. We have detected that incubation with BH_4 induces a small (ca 0.6 eV) upward shift in the edge position of the heme iron. This result is very exciting as it is the first indication that BH_4, though far away, affects the charge state and redox properties of the heme group.
5. The quality of the data was so good that preedge structure of the Fe K-edge could be resolved. The analysis is in progress.

In all, our data lend strong support to the supposition that the crystallographic data obtained on dimers of the heme subfractions are representative of the situation of the complete NOS dimers in working state as well. We are particularly excited about the observed effect of BH_4 on the edge position of the heme. Though this observation has not been understood yet, we hope that it will shed light on the mechanism through which the cofactor BH_4 can make NOS to synthesise NO rather than highly noxious superoxide.